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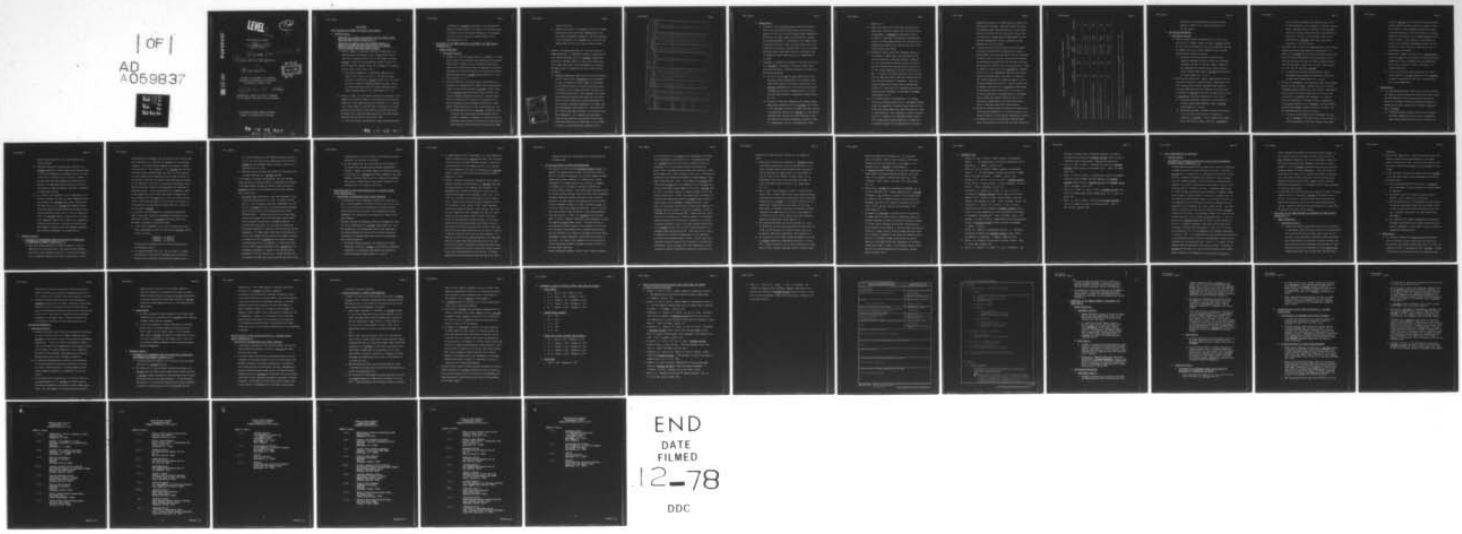

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6 Syphilis Vaccine and Immune Mechanisms.

by

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FINAL REPORT

I. BRIEF BACKGROUND AND SUMMARY OF RESEARCH ACCOMPLISHMENTS

A. ACQUIRED SYPHILIS

1. DEVELOPMENT OF AN EFFECTIVE AND PRACTICAL VACCINE AGAINST EXPERIMENTAL AND HUMAN SYPHILIS (OBJECTIVE NO. 1)

a. IMMUNIZATION OF RABBITS WITH LIQUID NITROGEN-PRESERVED, γ-IRRADIATED TREPONEMA PALLIDUM, NICHOLS STRAIN, IN A REDUCED INJECTION SCHEDULE (WITH AND WITHOUT ADJUVANTS)

1) Complete immunity to homologous challenge, accomplished by immunizing rabbits intravenously over a 37-week period with 60 injections containing a total of 3.71×10^9 freshly isolated *T. pallidum* γ-irradiated with 650,000 rads, formed the basis for studies under this contract toward the development of a practical vaccine (Miller, 1973; Miller, 1976).

   a) Short-term intramuscular or intravenous immunization of rabbits with a total of $12.3\text{-}18.7 \times 10^9$ liquid nitrogen-preserved, γ-irradiated *T. pallidum* divided into 3 or 4 doses and with or without an alginate-gluconate adjuvant, failed to provide significant protection against homologous intradermal challenge (Miller, 1976; Annual Reports No. 1-5).

2) Inasmuch as VDRL, TPI, and FTA-ABS antibodies are important in the diagnosis and control of syphilis, the finding that they develop during the 37-week immunization process referred to in 1) and persist in some animals for one year after vaccination, is of great significance and points to the serious restrictions which could be imposed upon a practical human vaccine with the same antibody-producing capacity (Miller, 1973; Miller, 1976).

   a) Each of the animals vaccinated with liquid nitrogen-preserved,

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γ-irradiated T. pallidum as described in 1a) developed both VDRL and FTA-ABS antibodies; in contrast, TPI antibody failed to develop in any of the animals immunized by the intramuscular route, with or without the adjuvant (Miller, 1976; Annual Reports No. 1-5).

2. MECHANISM(S) OF THE IMMUNE RESPONSE IN EXPERIMENTAL AND HUMAN SYPHILIS (OBJECTIVE NO. 2)

a. HUMORAL MECHANISM(S)

1) Experimental Syphilis

a) Evidence of a role for humoral factor(s) in immunity to experimental syphilis was provided by the demonstration that passive immunization of rabbits by daily intravenous injections of immune serum, significantly delays the appearance and markedly diminishes the severity and duration of lesions which develop after challenge with T. pallidum (Bishop and Miller I, 1976; Annual Reports No. 1-5).

b) Further evidence for an operative humoral mechanism(s) was provided by the demonstration of immune rabbit serum factor(s) capable of inactivating virulent T. pallidum, Nichols strain, in an in vitro-in vivo neutralization assay in which a mixture of treponemal suspension and serum is incubated anaerobically at 34°C and the virulence of the organisms in the mixture determined by the intradermal inoculation of normal rabbits (Bishop and Miller II, 1976; Annual Reports No. 1-5).

(1) Following infection with T. pallidum, a relatively close correlation was shown between the development and persistence of immunity to symptomatic re-infection and the appearance and persistence of neutralizing serum factor(s), thus offering a possible means for evaluating the immune

status of the host.

(2) Complete inactivation of treponemes in the test by immune serum required both heat-stable (immunoglobulin?) and heat-labile (complement?) components, and was accelerated by pre-incubation of the treponemes for 4 hours with non-immune serum, but not by 100 μg/ml of added lysozyme.

c) Inasmuch as designed experiments for the future relating to immune mechanism(s) in experimental syphilis incorporated the use of graded challenge doses of T. pallidum, studies were initiated to determine whether the use of such graded doses in the same rabbit affect lesion development by the smaller challenge inocula as compared to the use of single doses in one animal.

(1) Completed experiments suggest that (a) lesions developing from 10^6 inocula tend to accelerate lesion development at sites inoculated with lesser numbers of treponemes; (b) the natural course of the smaller-inoculum lesions is modified in such a manner that fewer ulcerate; and (c) when the maximum dose is 10^5 treponemes, the lesions which develop may significantly affect only the natural course of those lesions which develop from smaller inocula with only a minimally significant or no effect upon lesion development (Annual Report No. 5 for details of the 2 initial experiments and Table I for results of the third experiment). It is apparent that experiments necessitating a challenge inoculum must utilize single rather than graded doses of treponemes in a test animal in order to avoid misleading and confusing results.

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Table I. Comparison of Lesion Development in Rabbits Injected with Single Doses vs. Graded Doses of *T. pallidum*

LESIONS

		Development			Characteristics			
			Incubation period, days			Time to Ulceration, days		
T. pallidum/site		Lesions/ Total sites	Range	Mean[a]	Ulcerative Lesions/ Total Lesions	Range	Mean[a]	DF + Lesions/ Lesions Examined
Single Dose	10^6	15/15	3-4	3.3 ± 0.46	15/15	3-13	9.3 ± 1.03	9/15
	10^5	24/24	5-7	5.7 ± 0.64	24/24	19-25	21.0 ± 1.96	24/24
	10^4	24/24	9-11	10.0 ± 0.69	24/24	19-25	22.6 ± 1.39	24/24
	10^3	24/24	11-16	12.6 ± 1.79	24/24	23-29	26.4 ± 2.02	24/24
	10^2	24/24	17-22	17.7 ± 1.13	24/24	24-35	27.0 ± 6.15	24/24
	10^1	24/24	19-23	19.9 ± 1.21	21/24	26-41	31.4 ± 4.01	14/14
	10^0	15/24	19-23	21.4 ± 1.55	14/15	31-48	40.8 ± 5.34	15/15
Graded Dose	10^6	8/8	3-4	3.1 ± 0.35	7/8	12-18	16.3 ± 1.98	7/8
	10^5	16/16	5-6	5.1 ± 0.34[c]	12/16	14-19	17.3 ± 1.37[c]	8/12
	10^3	15/16	8-17	10.0 ± 2.33[c]	4/15	27-35	24.5 ± 0.58[c]	4/7
	10^2	14/16	11-18	15.0 ± 2.11[c]	4/14	29-35	29.5 ± 3.29[b]	4/4
	10^1	14/16	17-21	19.1 ± 1.38[c]	3/14	24-25	31.7 ± 3.06[b]	5/6
Graded Dose	10^5	16/16	5-7	5.9 ± 0.62	16/16	14-35	20.2 ± 6.55	12/15
	10^4	16/16	7-11	8.6 ± 1.45[c]	15/16	17-43	24.3 ± 8.47[b]	9/15
	10^3	16/16	8-15	11.6 ± 2.37[b]	11/16	21-48	24.1 ± 7.25[b]	9/14
	10^2	13/16	15-23	17.5 ± 2.33[b]	6/13	29-41	35.0 ± 5.37[c]	5/8
Graded Dose	10^6	8/8	3-4	3.4 ± 0.52	8/8	8-17	12.0 ± 3.96	8/8
	10^5	8/8	5-6	5.2 ± 0.46[c]	7/8	10-25	15.4 ± 5.74[c]	4/8
	10^4	8/8	5-7	6.5 ± 0.76[c]	5/8	17-19	17.4 ± 1.14[c]	1/5
	10^3	8/8	6-18	9.5 ± 5.74[c]	5/5	16-35	21.0 ± 8.15[b]	1/4
	10^2	8/8	9-17	13.9 ± 3.56[c]	2/8	29-35	32.0 ± 4.24[b]	2/4
	10^1	7/8	13-25	15.6 ± 4.24[b]	1/7	29	29.0 ± 0.0	1/2
	10^0	1[illegible]	1[illegible]	17.0 ± 0.[illegible]	0/1		-	-

[a]Mean ± Standard Deviation. [b]Not Significant, $p > 0.05$, t-test. [c]Significant, $p < 0.05$, t-test.

2) Human Syphilis

a) The results of the *in vitro-in vivo* neutralization experiments in the experimental disease opened up new avenues of approach to the study of possible humoral immune mechanism(s) operative in the human disease, and provided the rationale for determining whether (1) differences in the immune status of patients with early, latent, and late syphilis can be distinguished on the basis of neutralization titers; (2) similar heat-labile and/or heat-stable factor(s) are operative and (3) demonstrable heat-stable factor(s) are immunoglobulin in nature.

b) Experiments to determine the parameters necessary for survival of *T. pallidum* in the presence of unheated, normal human serum were considered a necessary prerequisite to definitive neutralization studies.

(1) The use of the *in vitro- in vivo* neutralization assay employing a final concentration of 90% unheated normal human serum resulted in the partial inactivation of *T. pallidum*, presumably due to cross-reacting, heat-stable treponemicidal factor(s) (immunoglobulins?) in concert with heat-labile factor(s) (complement?) (Annual Reports No. 3-5).

(2) The use of a 30% final concentration of unheated normal human serum incubated with 10^7 *T. pallidum*, then diluted to 10^4 treponemes just prior to rabbit injection, resulted in satisfactory survival of *T. pallidum*; in a preliminary experiment with unheated late latent syphilitic serum utilizing these successful parameters, evidence of specific treponemicidal activity was demonstrable (Annual

Report No. 4).

(3) Despite the success with a 30% final concentration of human serum, absorption studies were initiated with the non-pathogenic T. phagedenis biotype Reiter (TPR) in an effort to eliminate the non-specific treponemicidal activity of normal human serum and thus allow the use of maximum serum concentrations (greater than 30%) in the neutralization assay system.

(4) Preliminary studies showed that treponemal motility in the presence of normal human serum absorbed with TPR could be maintained at 86% after anaerobic incubation for 13.5 hours when the absorbed serum was utilized at 90% final concentration; absorption was carried out at $4^{o}C$ for 16 hours employing a 10% final concentration of TPR. In contrast, the motility was only 24% in the presence of unabsorbed serum in 90% final concentration and under the same conditions of incubation. These data strongly suggest that the heat-stable treponemicidal factor(s) is a cross-reactive, treponemal group antibody elicited by host-indigenous, non-pathogenic treponemes (Annual Report No. 5).

(5) In a further series of investigations, it was shown, utilizing the Staphylococcal Protein A radioimmune binding assay, that absorption of unheated normal serum with TPR as described above removes IgG antibody and does not result in the binding of C, while absorption at $37^{o}C$ for 1½ hours results in C binding to the treponeme-antibody complex; this finding takes on added significance, inasmuch as C is probably essential for the demonstration of specific

treponemicidal factor(s) in human syphilitic serum by the neutralization technique. Additional studies indicated that unheated normal serum absorbed with a final concentration of 10% TPR at 4°C for 30 minutes, retains C activity while still accomplishing the same degree of cross-reacting treponemicidal factor(s) absorption as does absorption at 4°C for 16 hours.

(6) A neutralization and motility assay was thus performed utilizing 75% and 90% final concentrations of normal serum absorbed with TPR as described at 4°C for 30 minutes. As shown in Table II, the data clearly show the efficacy of the absorption technique with the 75% final serum concentration assay; however, they also indicate that a slight but definitive inactivation of T. pallidum and a decrease in motility is still demonstrable when the final concentration of absorbed serum in the assay is increased to 90%, thereby suggesting the incomplete removal of cross-reacting treponemicidal factor(s). Despite the fact that no apparent inactivation of T. pallidum was demonstrable by the absorbed normal serum utilized in the assay at 75% final concentration, subsequent motility experiments clearly showed that complete absorption could not be consistently demonstrated at this serum concentration under the described conditions of TPR absorption.

(7) Studies to determine whether increasing the concentration of TPR used for absorption to 33 1/3% would result in the effective removal of cross-reacting treponemicidal factor(s) as measured by assays utilizing final absorbed normal serum concentrations of 75% and 90%, have been consistently

TABLE II

IN VITRO - IN VIVO NEUTRALIZATION ASSAY OF NORMAL HUMAN SERUM[1]

Normal Human Serum	Serum Concentration	Lesions/ Total Sites	LESION INCUBATION PERIOD[3] Mean[4] (days)	Range (days)	% Motility
Unheated, Unabsorbed	90	1/5	24.0 ± 0.0	-	0
	75	1/5	24.0 ± 0.0	-	0
Unheated, Absorbed[2]	90	3/5	14.3 ± 0.9	13-15	50
	75	5/5	14.2 ± 0.4	14-15	80
Heat-Inactivated (56°C-30'), Unabsorbed	90	5/5	13.4 ± 1.7	11-16	92
	75	5/5	13.4 ± 0.8	12-14	90
Heat-Inactivated (56°C-30'), Absorbed[2]	90	5/5	13.4 ± 0.8	12-14	96
	75	5/5	13.6 ± 0.8	12-14	92
Extract Control	90	5/5	13.4 ± 1.2	12-15	90
	75	5/5	13.8 ± 0.4	13-14	98

[1] Anaerobic incubation of normal human serum with 1×10^7 *T. pallidum*/ml for 16 hours at 34°C.

[2] Absorbed for 30 min. at 4°C with *Treponema phagedenis biotype Reiter* in 10% final concentration.

[3] 1×10^3 *T. pallidum*/site.

[4] Mean ± Standard Deviation.

successful, thus making feasible the assay of human syphilitic serum at one of these concentrations in the test system.

b. CELL-MEDIATED MECHANISM(S)

1) Experimental Syphilis

a) Efforts directed toward the demonstration of a direct cytotoxic effect of "immune" lymphocytes upon *T. pallidum* required the preliminary determination of the basic parameters necessary for adequate survival of normal lymphocytes and treponemes during suitable interaction periods.

(1) Although a successful system for measuring direct lymphocyte-treponeme interaction effects was developed, experiments designed to show that "immune" lymphocytes from experimentally-infected rabbits exert a specific, direct cytotoxic effect upon *T. pallidum* were unsuccessful (Annual Reports No. 1, 3-5).

b) Based upon the premise that "immune" lymphocytes must be preliminarily stimulated or "primed" before a demonstrable cytotoxic response can be evoked, experiments were conducted utilizing lymphocytes initially incubated for 24 hours with γ-irradiated *T. pallidum*, followed by the addition of virulent *T. pallidum* and continued incubation for 5 and 10 hours.

(1) Preliminary experiments indicated a killing effect of "primed" immune rabbit lymphocytes upon *T. pallidum* (Annual Reports No. 4 and 5).

(2) Experiments designed to confirm and extend the findings in (1) utilizing varying ratios of stimulator (γ-irradiated *T. pallidum*) to killer (immune rabbit lymphocytes) and killer to target (virulent *T. pallidum*), as

well as extended "priming" and incubation times, failed to show reproducibly clear evidence of a specific cytotoxic effect, although success was obtained in one experiment utilizing a "priming" time of 48 hours, stimulator/killer ratios of 1:1 and 5:1, a killer/target ratio of 100:1, 1000 virulent T. pallidum as the target, and aerobic incubation at 34°C for 10 hours.

c) On the basis that the use of an immunosuppressive agent causing lymphocyte depletion might enhance the susceptibility of rabbits to T. pallidum challenge and thus provide evidence for operative cellular mechanisms of resistance, rabbits were injected intravenously with goat anti-rabbit thymocyte globulin (ATG) and challenged, together with suitable control animals, with 10^6, 10^5, 10^4, and 500 T. pallidum.

(1) No significant differences were observed in lesion development between animals inoculated with ATG and the control animals (Annual Reports No. 4 and 5).

d) The role of macrophages in the syphilitic immune and pathologic process has been an enigma. Confusion exists as to whether macrophages become activated and, until recently, considerable doubt existed as to whether these cells (or any cells) had the capacity to phagocytize the treponeme. In a classic series of experiments, it has been possible to demonstrate the in vitro phagocytosis of T. pallidum by stimulant-induced peritoneal macrophages (Lukehart and Miller, In Press - See enclosed manuscript for technical details).

(1) Following a 4-hour incubation of macrophages with virulent T. pallidum at 37°C under 5% O_2, 5% CO_2, and 90% N_2 and in the presence of 20% heated immune serum, phago-

cytized T. pallidum can be visualized, following specific indirect immunofluorescent staining, as rounded, fluorescent "bodies" in the macrophage cytoplasm (See Figure 1 of enclosed manuscript); they are observed in increasing numbers as the duration of the treponeme-phagocyte interaction increases. Their presence is significantly reduced in the cytoplasm of macrophages treated with cytochalasin B, a known inhibitor of phagocytosis, and in non-phagocytic fibroblasts.

(2) Additionally, supportive evidence for T. pallidum phagocytosis in vitro has been provided by electron microscopic examination in which treponemes have been demonstrated within typical phagocytic vacuoles (See Figure 6 of enclosed manuscript).

(3) It was also shown that immune serum factor(s) significantly promote the in vitro phagocytosis of T. pallidum, although a contribution by heat-labile serum factor(s) has not been demonstrated.

2) Human Syphilis

a) As in the experimental model, efforts were directed toward the development of an assay system which would allow normal lymphocyte and treponemal survival during suitable interaction periods as a prelude to definitive experiments designed to demonstrate the direct cytotoxicity of "immune" lymphocytes upon T. pallidum.

(1) Although preliminary studies indicated that treponeme and normal lymphocyte survival could be accomplished under aerobic conditions for 5-10 hours in McCoy's 5a

medium (Annual Reports No. 2-5), these findings could not be confirmed.

(2) Completed studies have now shown that incubation of T. pallidum suspensions in M199 containing 20% human AB serum at 34°C in an atmosphere of 3% oxygen, results in no loss of motility or virulence for up to 20 hours. Further, it has been shown that human lymphocytes and monocytes suspended in the same medium remain viable for at least 49 hours under the same environmental conditions, as measured by trypan blue exclusion.

(3) As a result of the findings in (2), an experiment was conducted to determine the effect of normal lymphocytes upon the virulence of T. pallidum during their interaction at 34°C under 3% O_2; the T. pallidum and lymphocyte suspensions were prepared in M199 containing 20% human AB serum and were combined to give a 100 to 1 ratio of lymphocytes (10^6) to T. pallidum (10^4/ml). After 39 hours of incubation, lymphocyte viability was 97-99%, and no significant differences were observed in the time of lesion development compared to controls, when the incubated suspensions were injected intradermally into normal rabbits.

B. CONGENITAL SYPHILIS

1. DEVELOPMENT OF AN EXPERIMENTAL MODEL AND ELUCIDATION OF MECHANISM(S) OF PATHOGENESIS AND IMMUNITY (OBJECTIVE NO. 3)

a. Despite the rising prevalence of congenital syphilis, little is known about the interacting events, associated with the organism and the host, which determine infectivity. Ever since the recognition of congenital syphilis as a distinct clinical entity, it has

been accepted that treponemes cross the placenta only after the 18th week of gestation, at a time when the Langhans cell layer becomes atrophied. Yet, recent evidence suggests that placental transfer can occur earlier. The introduction of T. pallidum into the fetal circulation from an infected mother may result in a wide spectrum of events ranging from a fulminating, fatal disease to latency to the absence of infection; the factor(s) which influence the outcome are not understood. The lack of a satisfactory experimental rabbit model has precluded studies on the pathogenesis and immunology of congenital disease. Several investigators have attempted to demonstrate transmission of the disease from infected pregnant does to their offspring, both "in utero" and at birth, but without success. Further, conflicting data appear in the literature with regard to the susceptibility vs resistance of newborn rabbits to direct inoculation with T. pallidum.

b. As a prelude to the development of a satisfactory experimental model, studies were initiated to determine the susceptibility vs resistance of rabbits to T. pallidum shortly after their birth from normal, serologically non-reactive does.

1) In the initial experiment, 6 to 7 day old neonates were inoculated intradermally at each of 2 sites with T. pallidum, Nichols strain, as follows:

3 neonates - 5×10^3/site
3 neonates - 5×10^2/site
2 neonates - 5×10^1/site

Lesions failed to develop at any of the sites during the 46 day observation period.

2) In a second experiment with 6 to 7 day old rabbits, 3 animals inoculated at 2 sites with 10^3 treponemes per site failed to develop lesions during the 43 day observation period, while 3

of 5 sites inoculated with 10^6 organisms developed lesions in 5 to 14 days; the control adult rabbits which received 10^6 T. pallidum per site developed typical lesions at each of the 8 inoculated sites in 3 days.

3) These data clearly indicated that rabbits 6-7 days after birth are highly resistant to T. pallidum infection.

c. In an attempt to elucidate the mechanism(s) for this puzzling, innate resistance of neonatal rabbits, an experiment was designed to determine whether non-specific factor(s) which inactivate T. pallidum are present in both pregnant does and their offspring shortly after birth.

1) In vitro-in vivo neutralization assays were performed, as described by Bishop and Miller (II, 1976), on pooled sera from the 6 to 7 day old neonates inoculated intradermally with 10^3 and 10^6 T. pallidum per site in the above-described experiment as well as on the sera of the matching does prior to and during gestation. Neutralizing activity was clearly demonstrable in the pooled basal (pre-inoculated) serum obtained from the neonates the day prior to attempted infection. At one month post inoculation, at a time when the animals may have become susceptible to infection, neutralizing activity had disappeared. At 3 months post-inoculation, neutralizing activity was still absent from the serum of those animals injected as neonates with 10^3 T. pallidum per site; however, evidence of neutralizing activity was apparent at 3 months post-inoculation in those animals administered 10^6 T. pallidum per site as newborns, presumably in response to the infection which developed at 3 of the injected sites. Of equal interest was the absence of neutralizing activity from the basal sera of the

matching does, as well as from their sera obtained at 20 days gestation (~10 days prior to delivery).

2) The data suggest that the neutralizing factor(s) present in the newborn sera were not acquired through transplacental transfer. Indeed, the evidence appears to implicate colostrum as the source of T. pallidum inactivation components; the disappearance of neutralizing activity at 1 month post-inoculation would seem to eliminate milk as the source, inasmuch as the offspring were still nursing at this time.

C. PURIFICATION AND IN VITRO TISSUE CULTIVATION OF T. PALLIDUM, NICHOLS STRAIN (OBJECTIVE NO. 4)

1. PURIFICATION BY DISCONTINUOUS FICOLL DENSITY GRADIENTS

a. The preparation of pure treponemal suspensions is essential not only to the development of a practical vaccine, but also as a prelude to the isolation and characterization of proteins and/or polysaccharides which conceivably could be employed as either immunogen(s) for vaccination or as antigen(s) for specific serologic diagnosis.

b. Although a multiple centrifugation method was developed for obtaining "clean" suspensions of T. pallidum (Annual Reports No. 1-3), the improbability of obtaining unequivocally pure organisms by this method prompted full direction of our efforts toward the development of a technique based upon the use of discontinuous Ficoll density gradients.

1) Preliminary studies resulted in the separation of "clean" suspensions of motile T. pallidum, as determined by darkfield examination, but occasional tissue debris was observed by electron microscopy (Annual Reports No. 2 and 3).

2) It seemed apparent from the experiments in 1) that consistently effective separation of T. pallidum from rabbit host testicular tissue utilizing the density gradient technique, necessitated a determination of the true buoyant density of the organism; this was determined to be 1.190 to 1.93 gms/ml for T. pallidum, Nichols strain, utilizing linear sucrose gradients (Annual Reports No. 4 and 5).

3) Studies to develop a consistently reproducible Ficoll density gradient technique for purification of T. pallidum, based upon the buoyant density of the organism, were successful. Ten experiments have already been carried out in which the constructed discontinuous Ficoll gradients (1.085, 1.075, 1.065, 1.055 and 1.045 gm/ml) were altered from the initial experiments. Darkfield and electron microscopy examination of the four interdensity zones revealed "clean" single treponemes within the two upper interfaces (1.065-1.055 and 1.055-1.045) and some clumped and single treponemes plus some cell debris within the two lower zones (1.085-1.075 and 1.075-1.065). Further, by diluting material from the bands 1:3 with equilibrated 50% heat-inactivated normal rabbit serum in saline, it was possible to make the observation that 50%-60% of the organisms from the two upper bands were actively motile in contrast to 20% active motility within the two lower interfaces; it is important to note that the remaining 40%-50% of the treponemes from the two upper bands exhibited definitive but less than active motility which, based upon our unpublished observations, are in all probability as fully virulent when freshly isolated as are those with demonstrable active motility. Of further significance was the finding that more than 50% of the motile

organisms layered onto the gradient were recovered from the two upper zones.

2. IN VITRO CULTIVATION IN TISSUE CULTURE MONOLAYERS

a. The inability to either culture *Treponema pallidum in vitro* or to separate the organism from the rabbit host testicular tissue in which it is presently propagated, has seriously hampered and made complex those studies relating to 1) metabolism; 2) vaccine development; 3) serologic specificity in relation to diagnosis; 4) mechanism(s) of resistance; 5) antigenic structure and 6) physico-chemical make-up. While some limited knowledge has and will become available through studies utilizing tissue-contaminated treponemal suspensions prepared from rabbit testes, detailed, definitive, and more complete investigations can only be carried out with treponemes obtained as a result of either *in vitro* cultivation or large-scale separation techniques. Thus, it was felt that intense efforts should be applied in both these directions.

b. Since the discovery, in 1905, of *T. pallidum* as the etiologic agent of syphilis, numerous investigators have attempted to culture the organism *in vitro* without success. The great majority of investigations relating to growth of the organisms in tissue culture were made on the assumption that treponemal multiplication would be reflected by an increase in the number of actively motile (presumably virulent) organisms in the extracellular tissue culture fluid. As a result, this assumption led to the discard of culture exhibiting non-motile treponemes, usually within one to two days after inoculation.

c. During collaborative studies, with Dr. John A. Sykes, relating to

the ultrastructure of T. pallidum and the pathogenesis of disease in the experimental rabbit and in man, it was demonstrated that 1) T. pallidum takes up an intracellular residence within the parenchymal cells of the infected rabbit testes (Sykes and Miller, 1971); the infected labium (Sykes, Kalan and Miller, 1974); and the human cervix (Sykes and Kalan, 1975); and 2) ultrastructural differences exist between the pathogenic and non-pathogenic treponemes (Sykes and Miller, 1973). The finding of T. pallidum within tissue cells in vitro led to the suggestion that an in vitro intracellular relationship between mammalian cells and T. pallidum might be vital to its multiplication in a manner analogous to viral and bacterial replication. Further, the characteristic differences in ultrastructural morphology between T. pallidum (rabbit adapted or human strains) and non-pathogenic treponemes provide a useful marker for preliminary identification of replicating organisms in in vitro cultivation systems in which host-indigenous, avirulent treponemes may also become established. Studies were thus initiated with Dr. John Sykes and Dr. Thomas Fitzgerald (a postdoctoral research fellow now at the University of Minnesota) in an effort to explore the possible entry of the rabbit-adapted Nichols strain of T. pallidum into the cells of tissue culture monolayers with subsequent growth and multiplication. These have led to a series of cooperative and fruitful investigations in this laboratory (with Sykes and a graduate student, Steven J. Norris) and in the laboratory of Russell Johnson (with Fitzgerald) at the University of Minnesota; the key findings, which have formed and will form the basis for several investigations relating to cultivation and

pathogenesis in these and other laboratories, are summarized below:

1) Under aerobic conditions of incubation, T. pallidum attaches to and actively penetrates tissue culture monolayer cells of rabbit and human origin within 30 minutes after inoculation, survives significantly longer within or in the presence of the cells than in their absence (at least 4 days), and is demonstrable within the cells, by electron microscopy, for 7 days (Fitzgerald, Miller and Sykes, 1975; Annual Report Nos. 2-5).

2) When 0.5 mM-2.0 mM dithiothreitol (DTT) or 2.0 mM dithioerythritol (DTE) is added to MEM tissue culture extraction medium containing 50% fresh heat-inactivated normal rabbit serum (NRS), suspensions of T. pallidum in a cell-free system survive for a significantly longer period in the presence of 3% O_2 than under aerobic or anaerobic conditions of incubation (virulent treponemes were demonstrable for 10 days in the presence of 1.0 mM DTT and 3% O_2) (Norris, Miller, Sykes and Fitzgerald, submitted for publication; Annual Report No. 5).

3) MEM containing 50% fresh, heat-inactivated NRS and 0.5 mM-2.0 mM DTT has no effect upon the growth and multiplication of tissue culture monolayer cells (Norris, Miller, Sykes and Fitzgerald, submitted for publication; Annual Report No. 5).

4) The interaction of Sflep rabbit epithelial monolayer cells and T. pallidum suspensions in MEM medium containing 1.0-2.0 mM DTT, 1.2 mg/ml glutathione and 0.12 mg/ml cysteine in an atmosphere of 3% O_2, enhances treponemal survival (virulent trepo-

nemes were demonstrable throughout the 7 day observation period (Fitzgerald, Johnson, Sykes and Miller, 1977); these findings and those described under 2) lend support to the hypothesis that T. pallidum is microaerophilis.

5) T. pallidum attached to monolayer cells survives significantly longer than do unattached organisms (Fitzgerald, Miller and Sykes, 1975; Fitzgerald, Johnson, Sykes and Miller, 1977; Fitzgerald, Johnson, Miller and Sykes, 1977; Annual Report Nos. 3-5).

6) Heat-killed T. pallidum and non-pathogenic treponemes fail to attach to monolayer cells, thereby suggesting that T. pallidum attachment is not a phagocytic function of the cultured cells, but rather an active treponemal process associated with the pathogenic qualities of the organism (Fitzgerald, Miller and Sykes, 1975; Fitzgerald, Johnson, Miller and Sykes, 1977; Annual Report Nos. 3-5).

7) Attachment of T. pallidum to cultured cells can be prevented by immune syphilitic rabbit serum, as indicated by phase concontrast microscopy and rabbit inoculation; of special interest is the fact that a) blockage occurs without interfering with active motility of the organism, b) the heat-stable neutralizing factor(s) in immune serum described by Bishop and Miller (1976) may be similar or identical to the factor that prevents attachment, and c) blockage by immune serum may help to explain immunity to challenge and the early pathogenesis of the human disease which leads to latency, its persistence, and the occurrence of late disease manifestations (Fitzgerald, Johnson, Miller and Sykes, 1977).

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1. Bishop, N.H. and J.N. Miller. Humoral immunity in experimental syphilis. I. The demonstration of resistance conferred by passive immunization. J. Immunol., 117:191, 1976.

2. Bishop, N.H. and J.N. Miller. Humoral immunity in experimental syphilis. II. The relationship of neutralizing factors in immune serum to acquired resistance. J. Immunol., 117:197, 1976.

3. Fitzgerald, T.J., Miller, J.N. and J.A. Sykes. Treponema pallidum, Nichols strain, in tissue cultures - cellular attachment, entry and survival. Infect. and Immun., 11:1133, 1975.

4. Fitzgerald, T.J., Johnson, R.C., Sykes, J.A. and J.N. Miller. Interaction of Treponema pallidum (Nichols strain) with cultured mammalian cells: Effects of oxygen, reducing agents, serum supplements, and different cell types. Infect. and Immun., 15:444, 1977.

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influence of oxygen tension, sulfhydryl compounds, and serum on the motility and virulence of Treponema pallidum (Nichols strain) in a modified tissue culture medium. Submitted for publication.

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II. MAJOR ACCOMPLISHMENTS AND CONCLUSIONS

A. ACQUIRED SYPHILIS

1. DEVELOPMENT OF AN EFFECTIVE AND PRACTICAL VACCINE AGAINST EXPERIMENTAL AND HUMAN SYPHILIS (OBJECTIVE NO. 1)

a. Although complete immunity can be accomplished in rabbits by immunizing intravenously with 60 injections of freshly isolated, γ-irradiated *T. pallidum* over a 37 week period, no significant protection was achieved when the γ-irradiated treponemes were preserved in liquid nitrogen and used as immunizing agents, with or without an alginate-gluconate adjuvant, in a practical injection and time schedule. Failure to note protection may be a reflection of either 1) immunogen damage during the liquid nitrogen storage processing procedure, 2) immunogen damage during storage in liquid nitrogen, 3) the relative ineffectiveness of the alginate-gluconate adjuvant, and/or 4) the release of soluble surface immunogen(s) into the serum-saline glycerol supernatant during preparation of the treponemes for liquid nitrogen storage. The latter theory is consistent with the fact that freshly-isolated *T. pallidum* contains an "outer coat" at its periphery thought to be associated with virulence and which is lost rapidly *in vitro* upon aging. In the preparation of *T. pallidum* suspensions for liquid nitrogen storage, the 66 ml of γ-irradiated treponemes in 50% normal rabbit serum-saline containing 15% glycerol are centrifuged at 19,000 X g for 30 minutes and resuspended in 1 ml of the supernatant; the remaining supernatant is frozen at -20°C. It is conceivable that during either the irradiation or centrifugation process, soluble surface immunogen(s) detach from the organisms into the surrounding fluid. Indeed, it is possible that the complete immunity demonstrable with freshly-isolated, γ-irradiated *T. pallidum* may have been due to the injection of soluble surface immunogen(s) contained in the suspension injected the day of preparation

without high-speed centrifugation and liquid nitrogen storage. By this procedure, each of the animals received a total of 60 ml of fresh immunogenic suspensions, while in the experiments carried out with liquid nitrogen-preserved treponemes, each rabbit received a maximum of 4.8 ml of preserved suspensions which may have contained inadequate amounts of soluble immunogen(s). Thus, the results obtained during the tenure of this contract have pointed to the possibility that protection may be achieved with serum-saline-glycerol supernatant fluids (with and without complete Freund's adjuvant) obtained during preparation of γ-irradiated (650,000 rads) treponemes for liquid nitrogen storage.

b. Although VDRL and FTA-ABS antibodies were produced in rabbits following immunization with γ-irradiated *T. pallidum*, TPI antibody failed to develop. This points to the serious restrictions which could be imposed upon a practical vaccine with the same antibody-producing capacity, inasmuch as VDRL and FTA-ABS determinations are important in the diagnosis and control of the disease.

2. MECHANISM(S) OF THE IMMUNE RESPONSE IN EXPERIMENTAL AND HUMAN SYPHILIS (OBJECTIVE NO. 2)

a. HUMORAL MECHANISM(S)

1) Experimental Syphilis

a) Evidence that humoral mechanism(s) of resistance are operative in experimental syphilis has been provided by the demonstration of (1) passive protection by immune rabbit serum, and (2) a relatively close quantitative correlation between the development and persistence of symptomatic reinfection immunity following infection and the appearance and persistence of treponemicidal serum factor(s) measurable by an *in vitro-in vivo* neutralization assay involving heat-stable and heat-labile

components.

b) These studies suggest that a similar neutralization assay may provide a means for detecting differences in the immune status of patients with early, latent, and late syphilis, and thus allow a further understanding of the pathogenesis of the disease.

c) It has been shown that the use of graded doses of T. pallidum in the same rabbit affects lesion development by the smaller challenge inocula.

1) Lesions developing from 10^6 inocula tend to accelerate lesion development at sites inoculated with lesser numbers of treponemes.

2) The natural course of the smaller-inoculum lesions is modified in such a manner that few ulcerate.

3) When the maximum dose is 10^5 treponemes, the lesions which develop may significantly affect only the natural course of those lesions which develop from smaller inocula with only a minimally significant or no effect upon lesion development.

4) It is apparent that immunity experiments necessitating a challenge inoculum must utilize single rather than graded doses of treponemes in a test animal in order to avoid misleading and confusing results.

2) Human Syphilis

a) As a prelude to definitive in vitro-in vivo neutralization assays on syphilitic serum, studies have been carried out which showed that unheated, normal human serum, in a final concentration of 90%, is treponemicidal for T. pallidum. Although a satisfactory neutralization assay was developed utilizing

human serum in 30% final concentration, efforts were directed toward eliminating the non-specific treponemicidal activity so as to allow the use of maximal serum concentrations in the assay.

b) Absorption of normal human serum with the non-pathogenic T. phagedenis biotype Reiter was found to effectively remove treponemicidal activity, and should now allow the neutralization assay to be performed on syphilitic serum in final concentrations of 75% or 90%. Thus, the way is opened for exploring whether differences in the immune status of syphilitic patients can be elucidated on the basis of serum neutralizing activity.

b. CELL-MEDIATED MECHANISM(S)

1) Experimental Syphilis

a) Although a successful assay system was developed for determining any direct cytotoxic activity of "immune" lymphocytes upon T. pallidum, no such activity could be convincingly and reproducibly demonstrated. However, the results were suggestive enough to allow the conclusion that treponemicidal activity may be present on the surface of or within lymphocytes, but not completely accessible to the treponemes, thus opening up new avenues of approach based upon the use of disrupted lymphocytes.

b) No significant differences were observed in the development of lesions among rabbits inoculated intravenously with goat anti-rabbit thymocyte globulin, (an immunosuppressive agent causing lymphocyte depletion), as compared to the control animals.

c) It has been possible to demonstrate, for the first time, the in vitro phagocytosis of T. pallidum by stimulant-induced, peritoneal macrophages in the presence of heated immune rabbit serum. This lends support to the hypothesis that the macro-

phage may play a central role in the immune response to syphilitic infection, and provides the rationale for future studies directed toward the *in vitro* and *in vivo* demonstration of specific macrophage activation and subsequent *T. pallidum* inactivation and/or phagocytosis both in the experimental and human disease.

2) Human Syphilis

a) An assay system has now been developed in which normal human lymphocytes can be interacted with *T. pallidum* without exerting a harmful effect upon the treponemes.

b) It will now be possible to conduct experiments to determine whether whole or disrupted lymphocytes from patients with early, latent, and late syphilis exert a direct cytotoxic effect upon *T. pallidum*, and whether any demonstrable cytotoxicity can be correlated with their immune status. Such data will provide valuable information toward our understanding of syphilis pathogenesis.

B. CONGENITAL SYPHILIS

1. DEVELOPMENT OF AN EXPERIMENTAL MODEL AND ELUCIDATION OF MECHANISM(S) OF PATHOGENESIS AND IMMUNITY (OBJECTIVE NO. 3)

a. It has been shown that rabbits 6 to 7 days after birth are highly resistant to *T. pallidum* infection.

b. The serum of 6 to 7 day old rabbits exhibiting resistance to *T. pallidum* infection contain neutralizing factor(s) which inactivate *T. pallidum*, despite the absence of neutralizing activity from the matching does prior to and during pregnancy. At one-month post-inoculation, at a time when the animal may have become susceptible to infection, neutralizing activity had disappeared from the

offspring sera. The evidence appears to implicate colostrum as the source of T. pallidum inactivation components.

c. In addition to the elucidation of important information relative to the natural resistance of newborn rabbits, these investigations should provide the basis for experiments pertinent to the understanding of those factors necessary for the establishment of highly susceptible neonates as a prelude to the development of a congenital syphilis model in which transplacental transmission can be accomplished. Further, if a correlation can be established between neutralizing factor(s) and susceptibility vs resistance in the newborn and developing adult, the postulate that humoral mechanism(s) play a role in acquired resistance would be strengthened considerably.

C. PURIFICATION AND IN VITRO TISSUE CULTIVATION OF T. PALLIDUM, NICHOLS STRAIN, (OBJECTIVE NO. 4)

1. PURIFICATION BY DISCONTINUOUS FICOLL DENSITY GRADIENTS

a. A consistently reproducible Ficoll density gradient technique has been developed for separation of motile T. pallidum from rabbit host testicular tissue.

b. If further studies indicate that such gradient-derived, purified treponemes have retained virulence as well as ultrastructural and antigenic integrity, the avenues of approach will be opened for the in-depth analysis of the antigenic structure, immunogenicity, antigenic interrelationships among strains, and physico-chemical characteristics of T. pallidum not now possible with the relatively crude and/or altered treponemal preparations obtainable by present techniques. Investigations of this nature would ultimately lead to the isolation of immunogen(s) for vaccination and antigen(s)

for specific serologic diagnosis.

2. IN VITRO CULTIVATION IN TISSUE CULTURE MONOLAYERS

a. Although it has not as yet been possible to cultivate *T. pallidum*, *in vitro*, several important findings have been obtained which are pertinent not only to the problem of cultivation but also to the understanding of the pathogenesis of syphilis.

1) Under aerobic conditions of incubation, *T. pallidum* attaches to and actively penetrates tissue culture monolayer cells of rabbit and human origin within 30 minutes after inoculation, survives significantly longer within or in the presence of the cells than in their absence (at least 4 days), and is demonstrable within the cells, by electron microscopy, for 7 days.

2) When 0.5 mM-2.0 mM dithiothreitol (DTT) or 2.0 mM dithioerythritol (DTE) is added to MEM tissue culture extraction medium containing 50% fresh heat-inactivated normal rabbit serum (NRS), suspensions of *T. pallidum* in a cell-free system survive for a significantly longer period in the presence of 3% O_2 than under aerobic or anaerobic conditions of incubation (virulent treponemes were demonstrable for 10 days in the presence of 1.0 mM DTT and 3% O_2.

3) MEM containing 50% fresh, heat-inactivated NRS and 0.5 mM-2.0 mM DTT has no effect upon the growth and multiplication of tissue culture monolayer cells.

4) The interaction of Sflep rabbit epithelial monolayer cells and *T. pallidum* suspensions in MEM medium containing 1.0-2.0 mM DTT, 1.2 mg/ml glutathione and 0.12 mg/ml cysteine in an atmos-

phere of 3% O_2, enhances treponemal survival (virulent treponemes were demonstrable through the 7 day observation period); these findings and those described under 2) lend support to the hypothesis that T. pallidum is microaerophilic.

5) T. pallidum attached to monolayer cells survives significantly longer than do unattached organisms.

6) Heat-killed T. pallidum and non-pathogenic treponemes fail to attach to monolayer cells, thereby suggesting that T. pallidum attachment is not a phagocytic function of the cultured cells, but rather an active treponemal process associated with the pathogenic qualities of the organism.

7) Attachment of T. pallidum to cultured cells can be prevented by immune syphilitic rabbit serum, as indicated by phase contrast microscopy and rabbit inoculation; of special interest is the fact that a) blockage occurs without interfering with active motility of the organism, b) the heat-stable neutralizing factor(s) in immune serum described by Bishop and Miller (1976) may be similar or identical to the factor that prevents attachment, and c) blockage by immune serum may help to explain immunity to challenge and the early pathogenesis of the human disease which leads to latency, its persistence, and the occurrence of late disease manifestations.

b. The above studies provide excellent preludes and models for further studies relating to 1) in vitro cultivation in both cell-free and tissue culture systems, and 2) the elucidation of pathogenesis and immune mechanism(s) operative during the course of the experimental and human disease.

III. CHRONOLOGICAL INDEX OF TECHNICAL REPORTS ISSUED UNDER THE CONTRACT

A. ANNUAL REPORTS

1. No. 1: April 1, 1972 - March 31, 1973
2. No. 2: April 1, 1973 - December 31, 1973
3. No. 3: January 1, 1974 - December 31, 1974
4. No. 4: January 1, 1975 - December 31, 1975
5. No. 5: January 1, 1976 - December 31, 1976

B. PROGRESS REPORT ABSTRACTS

1. No. 1: 1972
2. No. 2: 1973
3. No. 3: 1975
4. No. 4: 1976
5. No. 5: 1977

C. RENEWAL APPLICATIONS (INCLUDES STATUS REPORTS)

1. No. 1: April 1, 1973 - December 31, 1973
2. No. 2: January 1, 1974 - December 31, 1974
3. No. 3: January 1, 1975 - December 31, 1975
4. No. 4: January 1, 1976 - December 31, 1976
5. No. 5: January 1, 1977 - December 31, 1977

D. FINAL REPORT

1. April 1, 1972 - December 31, 1977

IV. INDEX OF PUBLICATIONS AND NEAR-PUBLICATIONS ISSUED UNDER THE CONTRACT (3 Copies of each are enclosed)

A. Bishop, N.H. and J.N. Miller. Humoral immunity in experimental syphilis. I. The demonstration of resistance conferred by passive immunization. J. of Immunol., 117:191, 1976.

B. Bishop, N.H. and J.N. Miller. Humoral immunity in experimental syphilis. II. The relationship of neutralizing factors in immune serum to acquired resistance. J. of Immunol., 117:197, 1976.

C. Fitzgerald, T.J., Johnson, R.C., Miller, J.N. and J.A. Sykes. Characterization of the attachment of *Treponema pallidum* (Nichols strain) to cultured mammalian cells and the potential relationship of attachment to pathogenicity. Infect. and Immun., 18:467, 1977.

D. Fitzgerald, T.J., Johnson, R.C., Sykes, J.A. and J.N. Miller. Interaction of *Treponema pallidum* (Nichols strain) with cultured mammalian cells: Effects of oxygen, reducing agents, serum supplements, and different cell types. Infect. and Immun., 15:444, 1977.

E. Fitzgerald, T.J., Miller, J.N. and J.A. Sykes. *Treponema pallidum* (Nichols strain) in tissue cultures: Cellular attachment, entry, and survival. Infect. and Immun., 11:1133, 1975.

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H. Miller, J.N. Potential for vaccines for venereal diseases. Bull. of N.Y. Acad. Med., 52:no. 8, 986, 1976.

I. Norris, S.J., Miller, J.N., Sykes, J.A. and T.J. Fitzgerald. The influence of oxygen tension, sulfhydryl compounds, and serum on the motility and virulence of Treponema pallidum (Nichols strain) in a modified tissue culture medium. Submitted for publication. (Reprints will be sent upon receipt)

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19. KEY WORDS

I. Acquired Syphilis - Experimental and Human

A. Vaccine

1. γ-irradiated *Treponema pallidum*, liquid nitrogen preservation
2. Adjuvant
3. Homologous acquired resistance
4. Antibody response

B. Mechanisms of Immunity

1. Humoral

a. Passive protection
b. *In vitro-in vivo* neutralization
c. Single *vs* graded dose challenge

2. Cell-Mediated

a. Direct cytotoxicity
b. Anti-rabbit thymocyte globulin
c. Phagocytosis (Macrophages)

II. Congenital Syphilis - Experimental

A. Model Development
B. Mechanisms of Immunity and Pathogenesis

III. Purification - *Treponema pallidum*

A. Multiple Centrifugation
B. Discontinuous Ficoll Density Gradient

IV. *In Vitro* Cultivation in Tissue Culture Monolayers

20. ABSTRACT

I. ACQUIRED SYPHILIS

A. DEVELOPMENT OF AN EFFECTIVE AND PRACTICAL VACCINE AGAINST EXPERIMENTAL AND HUMAN SYPHILIS

Although complete immunity can be accomplished in rabbits by immunizing intravenously with 60 injections of freshly isolated, γ-irradiated *T. pallidum* over a 37 week period, no significant protection was achieved when the γ-irradiated treponemes were preserved in liquid nitrogen

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and used as immunizing agents, with or without an alginate-gluconate adjuvant, in a practical injection and time schedule; vaccinated animals produced both VDRL and FTA-ABS antibodies, but not TPI antibody.

2. The possibility exists that soluble surface immunogen(s) detach from the γ-irradiated treponemes during processing prior to storage, and that protection can be achieved with supernatant fluids obtained during vaccine preparation.

B. MECHANISM(S) OF THE IMMUNE RESPONSE IN EXPERIMENTAL AND HUMAN SYPHILIS

1. Humoral Mechanism(s)

a. Experimental Syphilis

1) Humoral mechanism(s) operative during the immune process have been demonstrated by the use of both passive protection and *in vitro*-*in vivo* neutralization techniques.

2) As a prelude to further immunity studies, it has been shown that the use of graded doses of *T. pallidum* in the same rabbit affects lesion development by the smaller challenge inocula. It is apparent that immunity experiments necessitating a challenge inoculum must utilize single rather than graded doses of treponemes in order to avoid misleading and confusing results.

b. Human Syphilis

1) Although a technique has been developed for determining the relationship of neutralizing antibody to immunity in patients, it involves the use of 30% unheated human serum due to the non-specific treponemicidal activity operative when a 90% concentration is employed.

2) Absorption of unheated normal human serum with non-pathogenic *Treponema phagedenis* biotype Reiter effectively removes the non-specific treponemicidal activity, and should now allow the neutralization assay to be performed on syphilitic sera in final concentrations of 75% or 90%.

2. Cell-Mediated Mechanism(s)

a. Experimental Syphilis

1) Although a successful assay system was developed for determining any direct cytotoxic activity of

"immune" lymphocytes upon T. pallidum, no such activity could be convincingly and reproducibly demonstrated. However, the results were suggestive enough to allow the conclusion that treponemicidal activity may be present on the surface of or within lymphocytes, but not completely accessible to the treponemes, thus opening up new avenues of approach based upon the use of disrupted lymphocytes

2) No significant differences were observed in the development of lesions among rabbits inoculated intravenously with goat-anti-rabbit thymocyte globulin (an immunosuppressive agent causing lymphocyte depletion), as compared to the control animals.

3) It has been possible to demonstrate, for the first time, the in vitro phagocytosis of T. pallidum by stimulant-induced, peritoneal macrophages in the presence of heated immune rabbit serum. This lends support to the hypothesis that the macrophage may play a central role in the immune response to syphilitic infection, and provides the rationale for future studies directed toward the in vitro and in vivo demonstration of specific macrophage activation and subsequent T. pallidum inactivation and/or phagocytosis both in the experimental and human disease.

b. Human Syphilis

1) An assay system has now been developed in which normal human lymphocytes can be interacted with T. pallidum without exerting a harmful effect upon the treponemes.

2) It will now be possible to conduct experiments to determine whether whole or disrupted lymphocytes from patients with early, latent, and late syphilis exert a direct cytotoxic effect upon T. pallidum, and whether any demonstrable cytotoxicity can be correlated with their immune status. Such data will provide valuable information toward our understanding of syphilis pathogenesis.

II. CONGENITAL SYPHILIS

A. DEVELOPMENT OF AN EXPERIMENTAL MODEL AND ELUCIDATION OF MECHANISM(S) OF PATHOGENESIS AND IMMUNITY

1. It has been shown that rabbits 6 to 7 days after birth are highly resistant to T. pallidum infection.

2. The serum of 6 to 7 day old rabbits exhibiting resistance to T. pallidum infection contains neutralizing factor(s) which inactivate T. pallidum, despite the absence of neutralizing activity from the matching does prior to and during pregnancy. The evidence appears to implicate colostrum as the source of T. pallidum inactivation components.

3. These investigations should provide the basis for experiments pertinent to the understanding of those factors necessary for the establishment of a highly susceptible neonate as a prelude to the development of a congenital syphilis model.

III. PURIFICATION AND IN VITRO TISSUE CULTIVATION OF T. PALLIDUM, NICHOLS STRAIN

A. PURIFICATION BY DISCONTINUOUS FICOLL DENSITY GRADIENTS

1. A consistently reproducible Ficoll density gradient technique has been developed for separation of motile T. pallidum from rabbit host testicular tissue.

2. If further studies indicate that such gradient-derived, purified treponemes have retained virulence as well as ultrastructural and antigenic integrity, the avenues of approach will be opened for the in-depth analysis of the antigenic structure, immunogenicity, antigenic interrelationships among strains, and physico-chemical characteristics of T. pallidum not now possible with the relatively crude and/or altered treponemal preparations obtainable by present techniques.

B. IN VITRO CULTIVATION IN TISSUE CULTURE MONOLAYERS

1. Under aerobic conditions of incubation, T. pallidum attaches to and actively penetrates tissue culture monolayer cells of rabbit and human origin within 30 minutes after inoculation, survives significantly longer within or in the presence of the cells than in their absence (at least 4 days), and is demonstrable within the cells, by electron microscopy, for 7 days.

2. When 0.5 mM-2.0 mM dithiothreitol (DTT) or 2.0 mM dithioerythritol (DTE) is added to MEM tissue culture extraction medium containing 50% fresh heat-inactivated normal rabbit serum (NRS), suspensions of T. pallidum in a cell-free system survive for a significantly longer period in the presence of 3% O_2 than under aerobic or anaerobic conditions of incubation (virulent treponemes were demonstrable for 10 days in the presence of 1.0 mM DTT and 3% O_2).

3. MEM containing 50% fresh, heat-inactivated NRS and 0.5 mM-

2.0 mM DTT has no effect upon the growth and multiplication of tissue culture monolayer cells.

4. The interaction of Sflep rabbit epithelial monolayer cells and T. pallidum suspensions in MEM medium containing 1.0-2.0 mM DTT, 1.2 mg/ml glutathione and 0.12 mg/ml cysteine in an atmosphere of 3% O_2, enhances treponemal survival (virulent treponemes were demonstrable throughout the 7 day observation period; these findings and those described under 2 lend support to the hypothesis that T. pallidum is microaerophilic.

5. T. pallidum attached to monolayer cells survives significantly longer than do unattached organisms.

6. Heat-killed T. pallidum and non-pathogenic treponemes fail to attach to monolayer cells, thereby suggesting that T. pallidum attachment is not a phagocytic function of the cultured cells, but rather an active treponemal process associated with the pathogenic qualities of the organism.

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8. Although it has not as yet been possible to cultivate T. pallidum, in vitro, the above findings provide excellent preludes and models for further studies along these lines, as well as for studies relating to pathogenesis and immunity.

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